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May 18, 2000

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FILING DATE: *April 01, 1999*
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By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

H. L. Jackson

H. L. JACKSON
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(b)(2).

Docket No. 99,101	Type a plus sign (+) Insid this box:	+

INVENTOR(S)/APPLICANTS(S)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (City and either state or foreign country)
Kapur	Ravi		Pittsburgh, PA
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Giuliano	Ken		Pittsburgh, PA

TITLE OF THE INVENTION (280 character maximum)

Design and Fabrication of Spatially Controlled Miniaturized Organ Systems from Stem Cells

CORRESPONDENCE ADDRESS

McDonnell Boehnen Hulbert & Berghoff
300 South Wacker Drive, Chicago

STATE	Illinois	ZIP CODE	60606	COUNTRY	U.S.A.

ENCLOSED APPLICATION PARTS (check all that apply)

X Specification Number of Pages 19 ☐ Small Entity Statement
☒ Drawing(s) Number of Sheets within specification ☐ Other (specify):

METHOD OF PAYMENT FOR THIS PROVISIONAL APPLICATION FOR PATENT

	A check or money order is enclosed to cover the Provisional Filing Fee.	PROVISIONAL APPLICATION FOR PATENT FILING FEE AMOUNT ($)	75.00
XX	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: **13-2490.**		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
__X__ No. _____ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,
SIGNATURE: _____ Date: 4/1/99

TYPED or PRINTED NAME David Harper REG. NO. 42,636

Additional inventors are being named on separately numbered sheets attached hereto.

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 99,101)

In re Application of:		
	Kapur et al.	Art Unit:
Serial No.:	To be Assigned	Examiner:
Filed:	Herewith	
For:	Design and Fabrication of Spatially Controlled Miniaturized Organ Systems from Stem Cells	

Asst. Commissioner for Patents
BOX PROVISIONAL APPLICATION
Washington, D.C. 20231

TRANSMITTAL LETTER

Sir:

1. We are transmitting herewith the attached papers for the above identified new provisional patent application:
   - ☒ Patent Specification (19 pages, including cover sheet, claims, and abstract)
   - ☒ Drawings (within specification)
   - ☒ Return Postcard
   - ☒ Other: Provisional Application Cover Sheet, Verified Statement claiming small entity status
2. ☐ A check in the amount of $75.00 is enclosed for the Filing Fee.

☒ Please charge the total filing fee of $75.00 to our Deposit Account No. 13-2490. A duplicate copy of this sheet is enclosed.

3. **GENERAL AUTHORIZATION TO CHARGE OR CREDIT FEES:** Please charge any additional fees or credit overpayment to Deposit Account No. 13-2490. A duplicate copy of this sheet is enclosed.

4. **CERTIFICATE OF MAILING BY "EXPRESS MAIL" UNDER 37 CFR § 1.10:** The undersigned hereby certifies that this Transmittal Letter and the paper, as described in paragraph 1 hereinabove, are being deposited with the United States Postal Service with sufficient postage as "Express Mail Post Office to Addressee" in an envelope addressed to: Asst. Commissioner for Patents, Box New Application, Washington, D.C. 20231, on this 1st day of April, 1999. Express Mail No. EM366140528US

By: _____

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PROJECT NARRATIVE

Part 4-a SCIENTIFIC AND TECHNOLOGICAL MERIT

Project Idea:
A major obstacle to the early stages of drug discovery is smarter lead compound optimization. We propose to develop an integrated solution that markedly shortens the drug discovery process by improving the decision making steps culminating with a selection of the most qualified lead compounds based on a miniaturized live-cell-based screening platform using novel fluorescent engineered biosensors.

We propose a platform that integrates several technologies that, until now, have been considered disparate and essentially unrelated. We will create a system wherein microarrays of selectively localized living cells, which contain engineered fluorescent biosensors, serve to integrate ultrahigh throughput screening (>100,000 compounds per day) and high-biological content screening onto a single platform. The high-biological content information obtained from probing target activity at a sub-cellular and molecular levels provides temporal-spatial dynamics of the drug-target interaction within each living cell. We believe that our revolutionary platform incorporating lead compound optimization assays that are automated, miniaturized, and information-rich will fulfill the need that the pharmaceutical industry has to dramatically improve their decision making process as well as their need to optimize lead compounds during the early part of the drug discovery process. Furthermore, we envision the shift of the industry away from isolated drug targets and the use of radioactive tracers toward the production and screening of combinatorial libraries containing millions of compounds using living organisms as test beds. Therefore, we view this shift as an opportunity to establish a new paradigm for drug discovery based on a marriage between fluorescence technology, micropatterning of living cells, automated optical detection and data analysis, and a new generation of bioinformatics approaches.

The technology developed through the proposed work will have a far reaching impact spanning the fields of drug discovery, biomedical research, environmental monitoring, and clinical diagnostics. The integrated platform with miniaturized organ-specific microarrayed cells capable of providing sub-cellular spatio-temporal information in response to drug-cell, toxin-cell, or pathogen-cell interactions will serve to enhance the decision making process in drug discovery, threat detection, and clinical diagnostics.

Innovation:

Bottlenecks in Early Drug-Discovery

Drug discovery can now be categorized as a series of processes that can be measured by the number of candidates identified in a given period with a defined level of resources. Productivity and speed become critical discovery performance metrics. The discovery process is now typically defined as being composed of four distinct, yet related, processes: (1) Target Identification/Validation, (2) Lead Identification, (3) Lead Optimization, and (4) Discovery/Development Interface. Early drug discovery

encompasses the first three of these processes. The successes in Target Identification due to the application of DNA sequencing and genomic databases have created serious bottlenecks downstream in the drug discovery pipeline (Figure 1). These constrictions exist at Target Validation, Lead Identification that includes ultra-high throughput screening (UHTS), and Lead Optimization. The pharmaceutical industry has eased some of these bottlenecks in Target Identification and Lead Identification by employing a brute force approach using UHTS and combinatorial chemistry with newly developed higher throughput tools. The critical constrictions at Target Validation and Lead Optimization still exist and can only be addressed by developing novel solutions to provide high content, or deep biology, information from live cell-based systems. Automation to speed and increase the volume of samples tested and data generation is alone insufficient to optimize the identification of lead compounds at the end of the early discovery pipeline prior to evaluation in animal models.

One of these critical bottlenecks, Lead Optimization begins with the identified "hits" from UHTS and focuses on the construction of a limited number of structural variations of the "hit" molecule coupled to biological evaluation for improvements in specificity, activity, selectivity and potency criteria. The rate at which "qualified leads "can be successfully generated from this process is dependent on the integration of chemical and biological data on each newly synthesized compound. Reduction in the time devoted to Lead Optimization can only be reduced by decreasing the number and length of feedback loops between chemists and biologists during analog synthesis and secondary biological testing.

Industry analysts have predicted that the pharmaceutical industry will need to increase the number of products launched by four fold to at least 24 to 36 drugs per annum earning more than $1 million dollars to achieve the forecasts for growth in global sales of 7% annually. The industry has reduced the average drug development project from 12 years to 8 years and is currently assessing and incorporating technologies that will reduce this to a period of 4 years in the future. With these reductions in development project duration, the drug discovery process becomes the major time consuming component of the entire drug discovery and development process. This fact requires that increases in efficiency and effectiveness be achieved to realize the promise of more interesting targets. Easing the bottleneck at Lead Optimization with technology capable of providing high content biological data for chemical compound evaluation can help achieve the target of reducing Lead Optimization from a process taking 24-36 months to a period of 12-18 months.

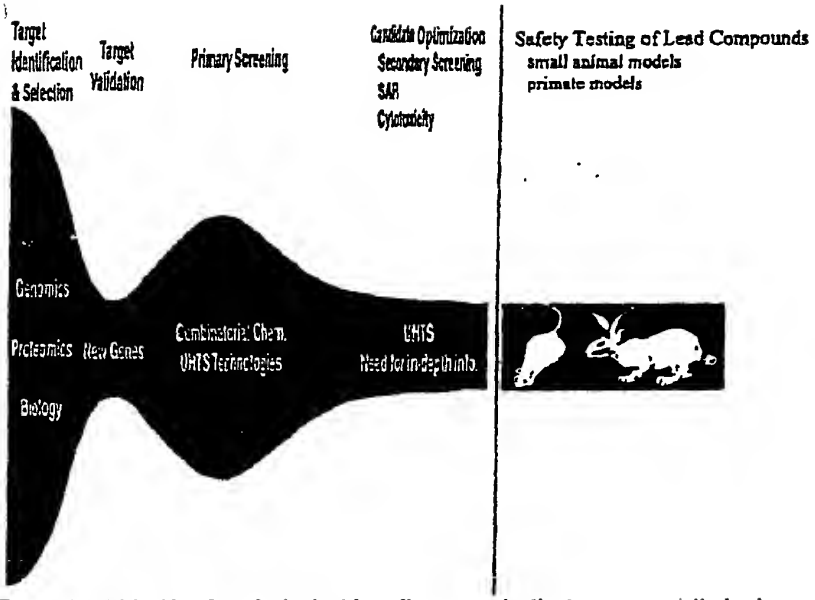

Figure 1. Critical bottlenecks in the 'drug discovery pipeline' can potentially lead to poorly qualified lead compounds being tested in animal models.

State of the art in early drug discovery

Radioactive assays and solution based fluorescence assays have dominated the primary screening phase of candidate compounds. These assays provide only limited information on the potency, and specificity of the candidate compounds because drug target activity is assessed using isolated sub-cellular components and not whole cells. Secondary screening in the candidate optimization phase may involve living cells that provide an average response that is either a fluorescence or radioactive read-out assay. These live cell assays provide an averaged population response from cells in 96 / 384 / 1536 microtiter plate to screen for target activity and cellular toxicity of the test compounds. The "hits" from these assays serve as binary indicators of the potency, target activity, toxicity, and bioavailability of test compounds leading to their classification as "lead compounds" for safety, toxicity, and efficacy testing in animal models. The absence of deep functional knowledge generation at the candidate optimization state, obtainable by the use of sophisticated, multi-color fluorescence cell based assays providing critical sub-cellular spatio-temporal target activity information, may result in "poorly qualified hits" being moved downstream into the animal testing phase. The brute force approach adopted by the pharmaceutical industry to deal with the dramatic increase in the size of chemical libraries is to employ ultra high throughput screening. However, this has only lead to a higher number of "poorly qualified hits" being passed down the pipeline towards expensive *in vivo* animal studies. In addition to UHTS or HTS it is therefore vital to provide a rich array of *in vitro* living cell based assays that provide "deep biology" on the specificity, toxicity, bioavailability, and potency of the candidate compound leading to better decision making in selecting the most optimum compounds

as lead compounds for further safety and efficacy testing in animal models.[1] This will greatly reduce the number of "poorly qualified hits" that find their way to animal testing before being qualified as "poor compounds". The optimization of "hits" and their classification into "highly qualified hits" or "poorly qualified hits" through live cell based assays that provide high content information will profoundly reduce the cost, time and ethical issues associated with extensive and unnecessary animal studies.

We propose a New Paradigm in Drug-Discovery

It is our perspective that a 4 pronged approach to smarter drug-discovery is needed to increase productivity by impacting the target validation and lead optimization points in the pipeline:

1) *High Content Screening* based deep biology information obtained from novel multi-parameter cell-based assays using advanced fluorescent protein biosensors. These novel assays will provide spatial and temporal information of target activity in, on and between cells. These assays used in conjunction with the state-of-the-art "averaged" cellular response assay will enable harnessing deep biology information to enable classification of "hits" into "highly-qualified" or "poorly-qualified".
2) *Miniaturization* of the live cell-based platform using novel microarrying techniques. Shrinking the cell-based screening platform onto a "chip" will enable generation of a combined UHTS and HCS platform thus increasing the speed and efficiency of data capture while reducing reagent volume and associated costs. Furthermore, developing "chips" microarrayed with organ specific cells leading to "organchips" will have a tremendous impact in screening the potency, specificity, toxicity, and efficacy of test compounds against a "tissue-like" ensemble leading to higher predictive relevance of the in-vitro live cell data. Deriving cells to be used as test beds for the compound candidates from pluripotent and/or totipotent stem cells is the ultimate vision for creating a stable and highly relevant in-vitro drug screening platform. Our approach to creating microarrays of an undetermined cell, followed by selectively induced controlled differentiation of the cells into organ specific cells is a very unique approach for creating chips with multiple cell types. We choose to use the genetic information coded in undetermined cells to guide them into a chosen differentiated state in specific locations on the chips. This approach to creating chips with multiple cell types is radically different from the existing state-of-the-art that utilizes a combinatorial approach to localize up to two immortalized cell types on a single substrate.[2a,2b]
3) *Automation* of assays using new advanced optical designs capable of resolving subcellular fluorescence signals in conjunction with robust automated hardware and software for data capture and analysis. The automation is critical to increasing the speed of screening the growing chemical libraries.

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[1] K. Giuliano et al. "High Content Screening: A New Approach to Easing Key Bottlenecks in the Drug Discovery Process", Journal of Biomolecular Screening, Vol. 2 (4), 1997.
[2a] S. Bhatia et al., JBMR
[2b]

4) *A bioinformatics* database to convert the data obtained from the multiparameter live cell fluorescence based assays within cells into new, deeper biological information and knowledge. The database is designed to capture the complex multidimensional interrelationships between biomolecules within cells by defining the temporal and spatial dynamics of intracellular molecular processes.

Our integrated approach to obtaining high content information from an automated miniaturized live cell-based test bed and converting the data into information and knowledge by creation of a informatics database is projected in Figure 2.

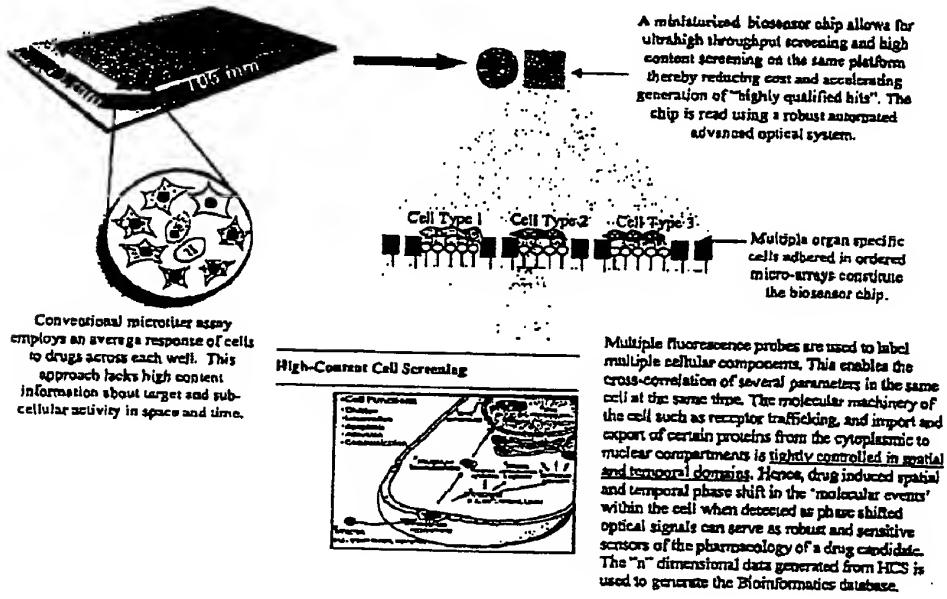


Figure 2. High Content Screening (HCS) and Ultra High Throughput Screening (UHTS) on a fully Automated Miniaturized Chip format using Organ specific cells yields deep biology knowledge of the spatio-temporal target activity in response to test compounds. The HCS information is used to populate and develop a Bioinformatics database to manage and analyze the multidimensional information that will originate from cell-based assays.

High Content Screening (HCS) as the Keystone of a New Paradigm

Cell function involves the dynamic distribution of biomolecules in the cell, in addition to changes in their activity. Developing an understanding of how targets

affect cellular function requires tools that provide temporal and spatial information of target activity within, on, and between cells. High Content Screens can be used to measure the effects of drugs on the complex molecular machinery such as signal transduction pathways, as well as on cellular functions like division, phagocytosis, endocytosis, exocytosis, locomotion, apoptosis, and cell-cell communication. We believe that HCS used in conjunction with novel fluorescent reagents, some of which are engineered to be expressed within living cells, will enable the automated extraction of multiparameter information from single cells and cell populations.

A significant component of HCS is the development of fluorescent protein biosensors designed to sense and report the intracellular spatio-temporal changes in target activity in response to a lead compound. Proteins have responded to evolutionary challenges to evolve into mediators and orchestrators of intracellular chemical reactions. As such, tagging them with fluorescent reagents enables their use as optical reporters of the dynamic distribution of specific reactions, kinetics of reactions, and post-translation modifications. The protein component of the biosensor serves as a highly evolved molecular-recognition moiety containing a fluorescent molecule attached in proximity to the active site of the protein acting to transduce the environmental changes into fluorescence signals for optical readout of target localization and activity. We have covered the basic principles and some initial applications of these fluorescent-protein biosensors in recent reviews.[2c, 2d] New fluorescent protein biosensors are depicted in Figure 3 along with other fluorescent reagents for HCS. Classes of these fluorescent reagents include labeling reagents that measure the distribution and amount of molecules in living cells as well as environmental indicators to report signal transduction events in space and time. Our approach is a multiparameter one that combines several key reagents in single cells to yield multidimensional information on the modulation of cell function by drug candidates. Our developmental efforts in engineering cells to express fluorescent-protein biosensors, where the fluorescent component is an autofluorescent derivative of the Green Fluorescent Protein (GFP), further strengthens HCS as the cells now express the biosensor(s) of use. We propose here to develop a novel class of HCS reagents to measure lead-compound-induced dynamic redistribution and alteration of the activity of intracellular constituents.

[2c] K. Giuliano et al., "Fluorescent protein biosensors: Measurement of molecular dynamics in living cells," Annu. Rev. Biophys. Biomol. Struct., Vol. 24, 405-434, 1995.
2d – Giulano and Taylor (TIBTECH)

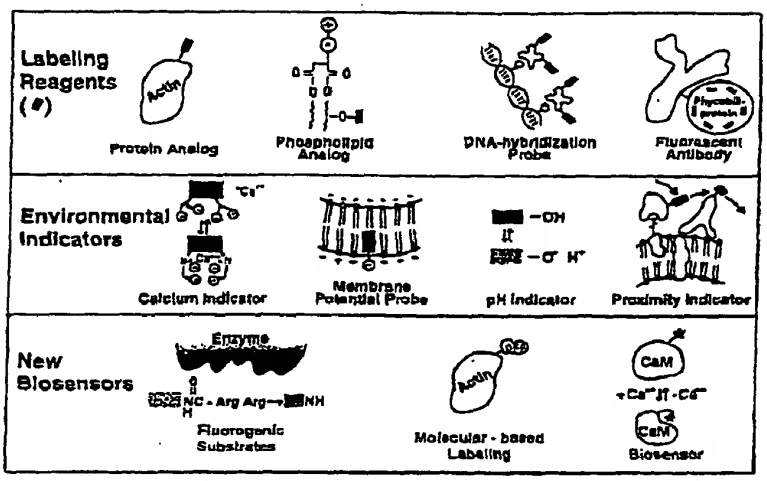


Figure 3. Fluorescent reagents for smart drug discovery.

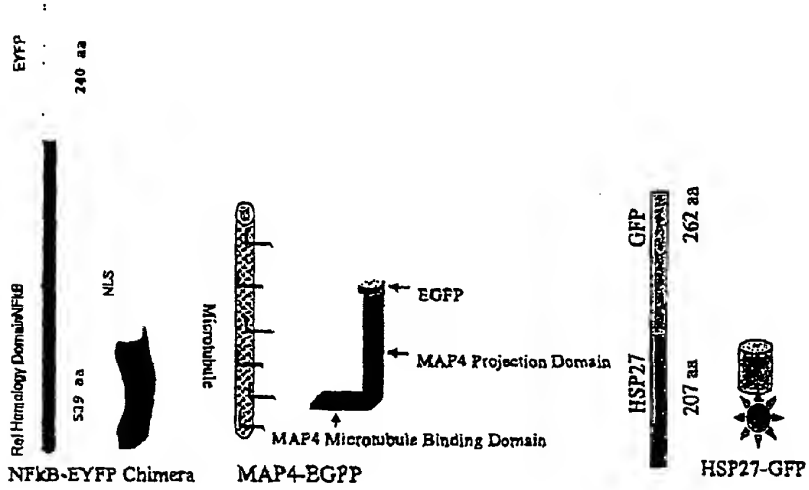


Figure 4. Novel high content screens to be developed are built on chimeras of key cellular proteins and green-blue-yellow-fluorescent proteins (GFP / BFP / YFP).

An example class of HCS involves the translocation of cytoplasmic ligand-receptor complexes, from the cytoplasm into the nucleus where transcriptional activation occurs (Figure 5).

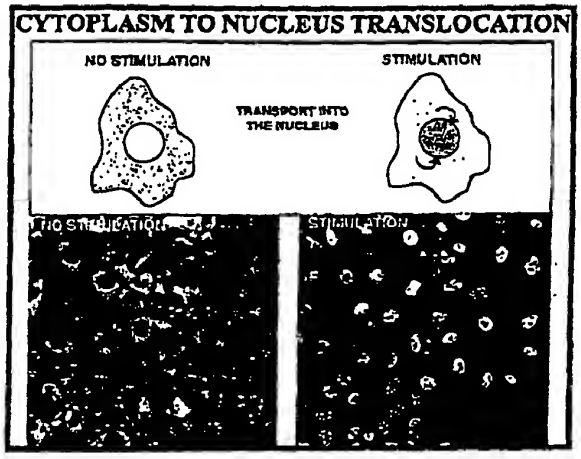


Figure 5. Measurement of drug induced redistribution of intracellular molecules. Custom written automated software enables characterization of cytoplasm to nuclear translocation of receptor-ligand complexes. The HCS in conjunction with our automated system can also provide kinetic (temporal) information of the intracellular redistribution of the relevant molecules.

Our novel proposal is to either transfect the undetermined cells with plasmids encoding for the protein chimeras, or transfect the differentiated organ specific cells with the plasmids. Transfecting each differentiated cell type with single or multiple plasmids encoding for protein chimeras will enable generation of cells that serve as their own reagents for high throughput and high content screens. This will enable generation of robust, knowledge based assays that are faster (no antibody staining steps) and more sensitive and specific (cross-reactivity of antibodies is no more an issue) than conventional cell-based fluorescence assays. We are the innovators of High Content Screening and are unaware of any others in the field who are working towards providing cell based High-Content Screens incorporating fluorescent protein biosensors.

Miniaturization

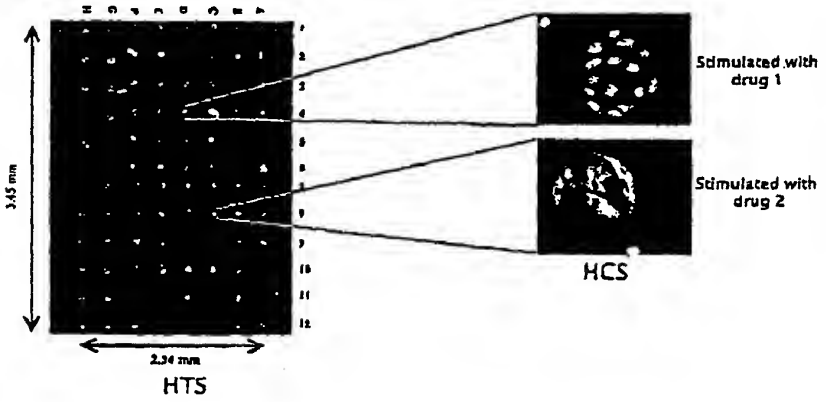
Miniaturization is one of the major driving forces involved in improving productivity in early drug discovery.[3,4,5] Our market research indicates that miniaturized cellular test beds with footprints the size of postage stamps will capture 50% of the microtiter plate market within 4 years. The advantages offered by miniaturization are: higher throughput, a combined HTS and HCS

[3] "High-Throughput Screening: Strategies and Suppliers" Report issued by HighTech Business Decisions, PO Box 6743, Moraga, CA 94570, June 3, 1998.

[4] R. Kapur et al., "CellChip™ System for Fluorescent Reporting of Toxins," GOMAC Proceedings, Monterey, CA, March 1999.

[5] R. Service, "Coming Soon: The Pocket DNA Sequencer", Science, Vol. 282, 400-401, 1998.

platform, reduced processing time, increased number of tests run in a massive parallel format on one substrate, and smaller reagent volumes translating to a dramatic reduction of cost and acceleration of productivity in candidate compound testing. For example, migrating assays from 96-well plate to 1536-well plate versions has dropped the reagent cost by 100 fold. Migration from 1536 to microarrayed cells on chips will further drop the cost and volume of reagents. Further, the ability to develop chips with multiple cells (organ or tissue specific cells) micoarrayed in preferred and know addresses will enable the use of these 'microscale organs' as powerful indicators and predictors of the in-vivo performance of the lead compound through High Content Screening. Developing these multiple cell based test-beds on plastics such as polystyrene and poly(ethylene teraphthalate) further adds the flexibility of using cheap disposable materials to reduce cost. We project that our miniaturized cell based combined HTS-HCS platform will march in parallel with other miniaturization technologies aimed to shrinking lab-top instruments into their hand-held miniaturized versions.[6,7] The combination of HTS and HCS on a single platform is depicted in Figure 6. This combined platform reduces the data capture, processing, and analysis time and provides a complete cell-based screening system. Developing technologies to enable arraying of multiple cells on the chip platform, with each cell carrying its own reagents in the form of our novel fluorescent protein biosensors, adds an n^{th} dimensional power to a complete drug screening platform. Furthermore, any reagent and assay technology developments made on today's platform will migrate directly to the next generation miniaturized platform.

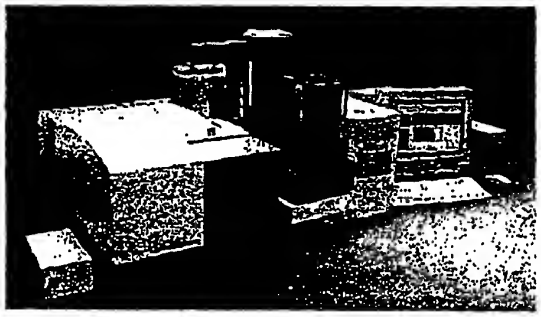


[6] Sid Marshall, "Fundamental Changes Ahead for Lab Instrumentation," R&D Magazine, Vol. 41 (2), February 1999.
[7] C. Wu, "The Incredible Shrinking Lab", Science News, 1998.

Figure 6. Combining HTS and HCS on the same platform. The massive parallelization achievable with miniaturization is shown in a simple simulation on this chip. The HTS is simulated here to detect "hits" on the miniaturized chip platform. Lack of fluorescence signals in wells H6, C9, C12, and D12, for example, indicate "non-hits". HCS measurements are then made only on the "hit" wells to gain more in-depth information to produce more "highly qualified hits". Further depth and breadth of information can be obtained by arraying multiple organ specific cells on a single chip and fluidically addressing each domain with a reagent of choice.

Automation

Automation is as vital a step in improving productivity of the drug discovery process as it was in increasing the throughput of genomic DNA sequencing. In keeping with our philosophy of increasing "speed and deep functional knowledge" to increase the productivity of primary and secondary screening of candidate compounds, we have focused our efforts in building an automated platform that supports multicolor fluorescence assays with up to four channels of fluorescence thus permitting multiparametric assays. The ArrayScan™ System is a fully automated platform for high-content screens performed in microtiter plates[8,9] and at least two pharmaceutical companies have included our technologies into recent publications. The instrument automatically scans microtiter plates and acquires multicolor fluorescence images of defined fields of cells at a predetermined sub-cellular resolution. The system contains robotic hardware for multiple microtiter plate handling, fluorescence excitation and emission optics, solid-state cameras, a dedicated processor, on-board algorithms for fluorescence image feature extraction, and database management capabilities (Figure 7). Additionally, there is an option to adapt a modular environmental-chamber with controlled temperature, humidity, and gas to enable kinetic analysis. Our automated platform captures 4 fields of view from each well, while analyzing the data in parallel, and completes the data capture and analysis of a 96 well plate in 25 minutes.



[8] G. Ding et al., "Characterization and Quantitation of NF-kB Nuclear Translocation Induced by IL-1 and TNF-a," The Journal of Biological Chemistry, Vol. 273 (44), 28897-28905, 1998.
[9] B. Conway et al., "Quantification of G-Protein Coupled Receptor Internalization Using G-Protein Coupled Receptor-GFP Conjugates with the ArrayScan™ High-Content Screening System," Vol. 4 (2), 73-84, in press.

Figure 7. The ArrayScan™ System. An automated platform produced by Cellomics™, Inc. for HCS using microtiter plates.

Bioinformatics

A database to convert the data obtained from the multiparameter live cell fluorescence based assays varying in space and time within cells into information and knowledge. The database is designed to capture the complex multidimensional interrelationships between biomolecules within cells by defining the temporal and spatial dynamics of intracellular molecular processes.

Productivity in the early drug discovery process in the post-genomic era is primarily limited by a lack of several components including appropriate assays, miniaturization, robust automation, and innovative hardware and software.[10] Our philosophy is to increase speed by robust automation and miniaturization, and to create knowledge by coupling high-content screens of lead compounds with a unique bioinformatics database.

We have developed several HCS reagents based on GFP and its mutants and have produced stable cells as reagents in a variety of immortalized mammalian cells of a single phenotype. Controlling the expression level of the chimera within the cells is the single most determining factor in moving from transient unstable clones to stable clones. Our attempts to transfect undetermined cells or their progeny will have more complex challenges. We will work to transfect the undetermined cells without inducing uncontrolled de-differentiation. Alternatively, we will transfect the progeny of the cells after their controlled differentiation on the chip.

Our innovative approach to patterning an undetermined cell type followed by selectively differentiating it into desired progeny on select regions of the substrate resulting in a chip with multiple cell types arranged in a pre-determined manner, requires 1) preventing uncontrolled differentiation of the undetermined stem cells post adhesion to the cytophilic regions, 2) selective addressing of the cytophilic regions with differentiating agents to enable controlled differentiation into the progeny of choice, and 3) transition of the technology from glass to commercially viable plastic substrates such as polystyrene. We believe that our approach to using model surfaces built on self assembled monolayers of thiols or silanes coupled to cell adhesive ligands will enable us to create cytophilic islands that enable adhesion of the undetermined cells while preventing their uncontrolled differentiation. Alternatively, we will cover the cytophilic islands with a feeder

[10] D. Kell, "Screensavers: Trends in High-Throughput Analysis," in Trends in Biotechnology, Vol. 17 (3), 89-91, 1999.

layer of fibroblasts incapable of mitosis. These feeder layers of fibroblasts have been used by others to control spontaneous differentiation of undetermined cells.[11] Furthermore, the use of automated precision instruments with a high degree of resolution, such as microspotters, will enable us to spot specific cytophilic regions with a differentiating agent of choice. These microspotters are currently being employed by the industry to automate the spotting of 1536 well microtiter format. Alternatively, we will engineer more sophisticated close-looped microfluidic cassettes that when interfaced with the chip will enable conduction of fluids of choice to specific locations on the chip.

We have developed ways of creating stable cytophilic islands on glass by using either thiol monolayers[12] or silane monolayers.[13] We are transitioning this technology to plastics, in particular polystyrene. We have to be able to surface modify the plastic without compromising its optical properties. Most surface modification processes for glass and silicon wafers are not amenable to plastics due to the nature of the harsh solvents used. Additionally, thiols are unsuitable for coating on plastics as they require a coinage metal for forming a coordination bond with the substrate. Silanes, though amenable to coating on plastics require a hydroxlayted surface, such as presented by glass and silicon, to form a covalent bond with the substrate. Additionally, the use of harsh solvents like toluene make it difficult to use silanes on plastics. However, our use of a low molecular weight PEG-silane and a hydrophobic silane, OTS, using methanol or ethanol based solvents, on polystyrene hydroxylated with a plasma discharge enables creation of stable cytophilic islands, surrounded by cytophobic regions. We project that this technology is adaptable to any plastic of choice by standard modifications of the proposed techniques.

Technology Development

We propose the following experimental paradigm (Figure 8):

1) Transfect 3 undetermined cell lines, such as SNB19, C2C12, and Ntera-12 with chimeras such as NF-kB/GFP, MAP-4/EGFP, HSP-27/BFP.

2) Pattern the transfected undetermined cells as single or mixed cultures on the glass and polystyrene chips bearing 100 μm – 500 μm diameter circular cytophilic islands separated by edge-to-edge spacing ranging from 25 μm – 125 μm. The overall footprint on the chip will match the format, and be a 150 fold smaller in surface area, of the 1536 (32 rows x 48 columns), 384 (16 rows x 24 columns), and 96 well (8 rows x 12 columns) microtiter-plates.

3) Differentiate the transfected patterned cells into single progeny (SNB19 to Glial; Ntera-2 to Neurons) or multiple progeny (C2C12 to Adipose and Skeletal Muscle Cells) using the appropriate differentiating agents applied either

[11] Cite the "Techniques In Cell Biology" manual
[12] Cite the CellChip proceedings abstract in GOMAC
[13] R. Kapur et al., "Cellular and Cytoskeletal Morphology.....ECR paper

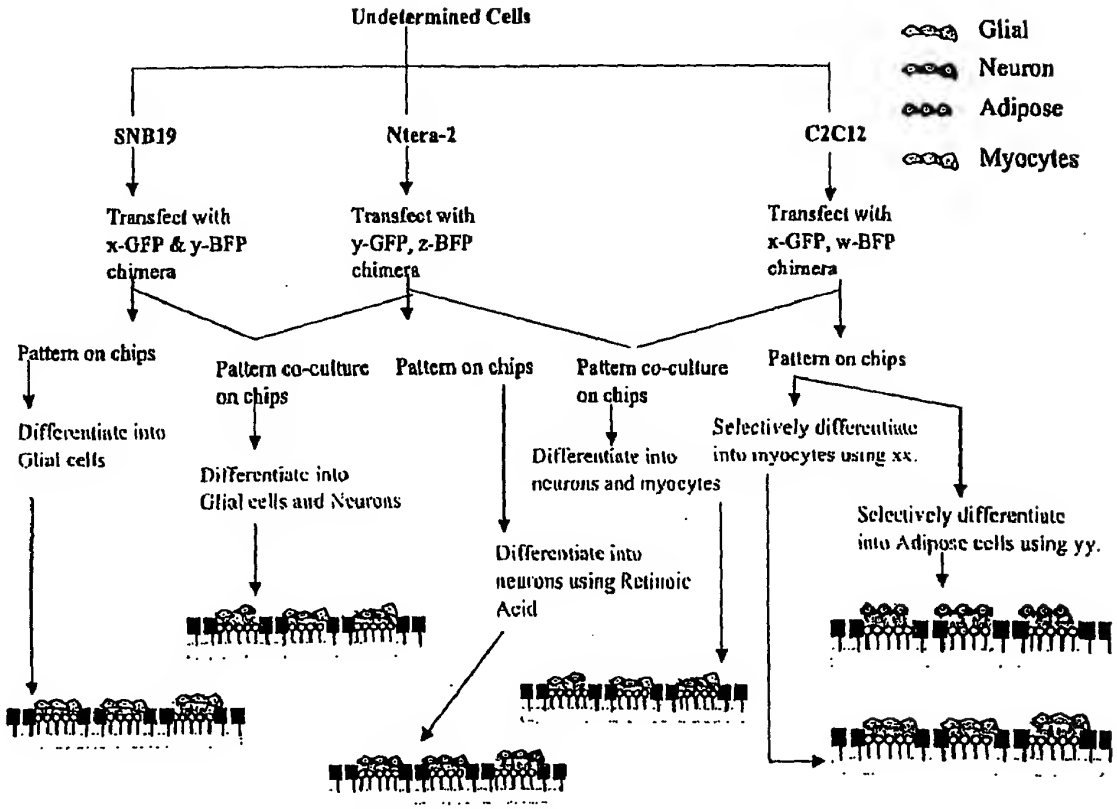


Figure 8. Experimental Flowchart. We will attempt to create chips that model the Brain tissue (co-culture of Glial and Neurons), Adipose and Skeletal Muscle tissue and Nerve and Muscle tissue interaction (co-culture of neurons and myocytes).

homogeneously to the chip (for single progeny) or selectively to specific cytophilic islands (multiple progeny). The co-culture of SNB19 and Ntera-2 and their differentiation into Glia and Neurons (the 2 dominant cell types in the brain) will enable the formation of chips with cytophilic islands that bear both glia and neurons in juxstaposition.

4) Determine the ability of the chips to serve as functional platforms for High-Throughput and High-Content Screening of drugs.

These studies will culminate with the coupling of Embryonic Stem (ES) cell technology to chips containing living organotypic tissues for drug discovery. In short, we propose to use simple to moderately complex models of cellular differentiation to provide a framework for the *parallel* development of fluorescent protein biosensors, and the organotypic cell chips for this next-generation drug discovery platform.

We will also develop several models of cellular differentiation as well as the parallel creation of fluorescent protein biosensors for use on our new drug discovery platform. In one model system, neuronal and glial precursor cells will be engineered to express fluorescent protein biosensors to measure the dynamics of their cytoskeletal proteins. The cytoskeleton has become a well-characterized and valid drug discovery target for which there are likely to be many lead compounds in the discovery pipeline at any one time. Each of the cell lines will express spectrally distinct biosensors such that they can be patterned into separate locations within a microarray as well as be patterned together, or cocultured, within the same location. The latter aspect is particularly exciting because we will be able, for the first time, to measure the simultaneous drug response of the two cell types in an organotypic context where the cells are allowed to interact as they would within the brain tissue of a living animal. Because the biosensors contained within each cell type will be spectrally distinct, the platform we develop will be able to detect and assign function to each cell type within the coculture.

In a second model system, a pluripotent cell line, mouse C2C12 cells, will be engineered with a fluorescent protein biosensor, patterened into microarrays, and differentiated into two cell types, skeletal muscle myocytes and adipocytes. In this case, the precursor cells will be engineered to express a fluorescent protein biosensor of carbohydrate metabolic flux that will be measured in both time and space within each cell. For muscle and adipose cells, carbohydrate metabolism plays an important role in regulating the physiological function of each cell type; contraction and relaxation of the muscle cells and fat storage and mobilization in adipocytes cells. Therefore, this model system will become a platform on which to measure the effect of lead compounds on the same molecular pathway within two tissue types. Moreover, the multiple tissue type screening platform proposed here permits the pharmaceutical industry to efficiently address several

fundamental tenets of the drug discovery process, the precise measurement of lead compound efficacy, specificity, and toxicology.

Finally, a third, more complex model system will be constructed from components of the first two models. We propose to co-culture neuronal and skeletal muscle precursor cells with both types being engineered to express spectrally distinct fluorescent protein biosensors. The cells will be patterned and differentiated on a chip. The effect of lead compounds on the complex interaction of neurons and skeletal muscle cells will be measured using the fluorescent protein biosensors engineered into each cell type. If the proper conditions for making the chip with these two distinct engineered sensor cells are found and exploited, we predict that the co-culture of differentiated neuronal and muscle cells will become a system in which we will be able to measure directly excitation-contraction coupling events and the effects that lead compounds have on these events. This approach, when generalized to interactions between other tissue types and interactions between multiple cell types within an organ will bring lead optimization to a level of sophistication unheard of in the pharmaceutical industry.

Organotypic Differentiation Model Systems

Glial differentiation. Cells taken from a highly aggressive human glioblastoma tumor have been shown to grow indefinitely in culture and to exhibit altered morphological and growth characteristics in the presence of a differentiation agent. These cells, named SNB-19 (Welch, et al., 1995), will be engineered to express a fluorescent protein biosensor (see below) and patterned onto chips either by themselves or in combination with neuronal precursor cells (see below). To induce differentiation, a mixture of dibutyryl-cAMP and isobutylmethyl xanthine (a phosphodiesterase inhibitor) will be added to the culture and the cells allowed to incubate for 12-24 h. These agents induce the cells to elaborate multiple processes that often interact with other glia in the same culture (Welch, et al., 1995) as well as cause the cells to stop dividing.

Neuronal differentiation. Several neuronal precursor cell lines exist that could possibly be tested in the new drug discovery platform we are developing. We will first test a well-characterized model system, NT2 cells from a human teratocarcinoma cell line (Pleasure, et al., 1992). These cells are unique in that they can be induced to differentiate into stable, post-mitotic human neurons and they have already been shown to be a vehicle for the expression of diverse gene products (Pleasure, et al., 1992). As with the glial cell precursors, we propose to engineer the precursor NT2 cells to express a fluorescent protein biosensor (see below), pattern them onto chips either by themselves or in a co-culture with precursor SNB-19 cells, and induce them to differentiate on the chip. Briefly, the precursor NT2 cells expressing the biosensor will initially enter a program of differentiation that begins with a two week treatment of retinoic acid, mitotic inhibitors, and a specialized extracellular matrix. The partially differentiated cells will be transferred to the chips where they will undergo the final stages of differentiation by elaborating processes that form axons and dendrites. The cells

will become post-mitotic but will retain the ability to express functional proteins such as the endogenous fluorescent protein biosensor.

Mixed glial-neuronal differentiation. NT2 precursor cells in the final stages of differentiation will be added along with both SNB-19 cells to the same chip. After both cell types attach, the co-culture will be treated with dibutyryl-cAMP and isobutylmethyl xanthine. The two cell types will be allowed to interact as they differentiate. Because the NT2 precursors are by this time committed to differentiation, we predict that the dibutyryl-cAMP added to the co-culture will have little or no effect on neuronal cell differentiation. It may even enhance neuronal cell differentiation because cAMP is generally known to induce the differentiation of several cell types.

Adipose and skeletal muscle tissue from a common precursor. The mouse C2C12 cell line is pluripotent and has been shown to be capable of differentiating into skeletal muscle (Cuenda and Cohen, 1999), adipocytes (Teboul, et al., 1995), and osteoblasts (Nishimura, et al., 1998). The precursor C2C12 will first be engineered to express a fluorescent protein biosensor of carbohydrate metabolism (see below). The cells will be patterned onto chips where the growth medium will contain < 1% calf serum. This large decrease in serum concentration (10% originally) induces the C2C12 precursor cells, over a period of 24-48 h, to stop dividing, fuse into multinucleated and elongated cells, and form contractile myotubes. To induce engineered C2C12 precursor cells to differentiate to adipocytes, the cells on chips will be treated with a mix of thiazolidinedione and fatty acid (Teboul, et al., 1995). The differentiation occurs after 24-48 h and is accompanied by the slowing of cell growth and the uptake of fatty acids by the cells and their incorporation into cytoplasmic lipid droplets.

Nerve and muscle tissue interaction. In this model system we will take cellular components from the other two model systems and combine them into a model of tissue-tissue interaction. The neuronal NT2 precursor cells will be combined with the muscle C2C12 precursor cells to allow them to interact during differentiation, much like tissues interact during normal development. Three scenarios will be tested: 1) NT2 and C2C12 precursors will be arrayed together on a chip and allowed to differentiate together; 2) NT2 precursors will be arrayed on a chip and differentiated, followed by the addition and differentiation of muscle C2C12 precursors; and 3) Muscle C2C12 precursors will be arrayed on a chip and differentiated, followed by the addition and differentiation of neuronal NT2 precursors. We will choose the model that yields the optimal neuron-muscle interaction data, judged morphologically, for future development.

Fluorescent protein biosensors

Introduction. It has now been two decades since our group realized the potential of fluorescence based reagents and methodology as applied to answering fundamental cell biological questions. Our most highly evolved approach to measuring chemical and molecular events in time and space within living cells involves the design and construction of fluorescent protein biosensors. Because proteins mediate nearly every chemical event within the living cell, we have taken advantage of the exquisite specificity, in terms of activity and intracellular localization, that proteins exhibit in performing their life sustaining functions. In

short, we have coupled the flexibility and sensitivity of fluorescence detection with the specificity of proteins to produce biosensors that report both protein dynamic distribution and activity within living cells. We have published reviews of fluorescent protein biosensor theory, design, and applications (Giuliano, et al., 1995, Giuliano and Taylor, 1998). We propose here to produce three novel fluorescent protein biosensors for use in the new drug discovery platform.

Neuronal cytoskeleton – BFP-β-tubulin chimera. The microtubule cytoskeleton has long been a valid drug target. We propose to design a fluorescent protein biosensor, which cells will produce themselves, to measure the dynamic assembly state of microtubules in living cells and use it to report changes in the microtubule cytoskeleton induced by lead compounds. First, we will access the cDNA encoding β-tubulin, one of the component proteins of the microtubule cytoskeleton, and fuse it to a cDNA coding for a blue fluorescent protein variant to form a BFP-β-tubulin chimera. When stably expressed in the NT2 precursor and differentiated cells, the chimera will become a biosensor of microtubule assembly. We will produce stable NT2 precursor cells expressing the BFP-β-tubulin biosensor, differentiate them to neurons, and treat them with drugs known to affect the microtubule cytoskeleton. Using the temporal and spatial image date from these experiments, we will construct computer algorithms that perform automatic feature extraction on a cell-by-cell basis to produce numerical data that quantify the state of microtubule cytoskeleton assembly in living drug-treated cells.

Glial cytoskeleton – GFP-GFAP chimera. Using an approach similar to that described above for the microtubule fluorescent protein biosensor, we will generate a green fluorescent chimera of the glial fibrillary acidic protein (GFAP), a component of the intermediate filament cytoskeleton. GFAP is a major cytoskeletal protein found in glial precursor cells and differentiated glia. The production of the GFP-GFAP chimeric protein will permit us to measure specifically the dynamics of the intermediate filament cytoskeleton (green) and the state of the microtubule cytoskeleton in neurons (blue) while the two cell types are being co-cultured.

Carbohydrate metabolism – PFK-2 phosphorylation sensor. Intermediary metabolism lies at the heart of myriad molecular processes within living cells. Until the advent of fluorescent protein biosensors there was no real hope of building a sensor that could provide a temporal and spatial map of the metabolic state of living cells. We propose to build a fluorescent protein biosensor that can be used to report the state of carbohydrate metabolism in living cells that have been treated with lead compounds. This sensor will find immediate application in our proposed muscle and adipose cell model systems since the regulation of energy metabolism in both these cell types is tightly coupled with cell function.

The enzyme we have chosen as the basis for our metabolic biosensor is 6-phosphofructo-2-kinase/Fructose-2,6-bisphosphatase (PFK-2). PFK-2 plays a key role in balancing cellular energy utilization and storage. That is, the activity of this bifunctional enzyme can act to switch a cell between carbohydrate oxidation (energy yielding) and carbohydrate synthesis (energy requiring). The key to building a biosensor out of PFK-2 is directly related to how its enzymatic activity is regulated through post-translational modification. The phosphorylation state of

PFK-2 dictates whether the enzyme will stimulate cellular carbohydrate breakdown or its synthesis (Kurland and Pilkis, 1995). We propose to exploit this very specific protein modification to build our biosensor. We will insert the amino acid sequence containing the PFK-2 phosphorylation site into various points within the coding sequence for GFP. Because the high resolution 3D-structure for GFP is known, we can precisely insert the PFK-2 phosphorylation site into what we believe are conformationally sensitive sites within the fluorescent protein. We will probe the GFP molecule at multiple points until we find those sites that alter the fluorescence properties of the protein when the inserted regulatory site of PFK-2 becomes phosphorylated. By measuring the fluorescence properties of the biosensor in time and space within a cell we will produce a dynamic map of PFK-2 phosphorylation, and therefore PFK-2 activity. Because PFK-2 activity is key to regulating carbohydrate metabolism, the biosensor we build will provide a direct readout of an essential molecular process within multiple cell types arrayed on chips.

Patterning of cells and reagent delivery

Fabrication of glass substrates with cytophilic islands. We will exploit thiol based chemistries to fabricate chips on glass substrates. As thiols form coordination bonds with coinage metals, we will evaporate a thin layer (12 nm) of gold on glass substrates (10 mm x 10 mm). This will be followed by application of a 2 mM solution of a hydrophobic thiol, such as but not limited to hexadecanethiol or octadecanethiol, using either an ink-jet printer, textured elastomeric stamps, or by microspotting. We have used all three methods of transfer, with varying degrees of transfer fidelity, and will primarily focus on transfer by stamping or microspotting. The "footprint" on the chips will resemble that of a 1536, 384, and 96 well microtiter plate. The shape of the cytophilic islands will be circular, with a diameter ranging from 100 μm – 500 μm, and the inter-island edge-to-edge spacing will be controlled from 25 μm – 150 μm. We will establish the preferred "footprint" size (cell morphology and function will serve as metrices of success- we will aim for the smallest dispersion in phenotype and practically no dispersion in genotype as metrices of optimization) and develop chips with the optimized footprint. Post transfer of the hydrophobic thiol, the remaining uncovered regions on the gold coated glass substrates will be modified with a 2 mM solution of poly(ethylene gycol) terminated thiol (PEG-thiol). The hydrophobic islands of thiols surrounded by the hydrophilic 'sea' of PEG-thiol will be primed with cell adhesive extracellular matrix protein (fibronectin, laminin, or collagen) at ~25 μg/ml or the cell adhesive tri-peptide RGD at similar concentration. The protein or peptide will bind to the hydophobic regions (hexadecane thiol) while resisting adsorption onto the hydrophilic regions (PEG-thiol). This in turn will convert the hydrophobic regions into 'cytophilic' regions that are conducive for binding cells.

Fabrication of plastic substrates with cytophilic islands. The chemistry of organosilanes will be utilized to form regions of cell adhesive and cell repulsive cues on oxidized polystyrene. Atactic and tactic polystyrene will be oxidized (with a radio frequency glow discharge oxygen plasma) to present

surface hydroxyl groups which will react with the organosiles to produce covalent Si-O-substrates (siloxane) linkages. Post oxidation, a micropatterned stencil (such as an SEM grid or micropatterned poly(dimethyl) siloxane) will be coupled onto the polystyrene surface. A liquid hydrophobic silane, such as but not limited to, dichlorodimethylsilane or OTS with a relatively high vapor pressure will be vapor deposited onto the surface of the masked polystyrene for 15 minutes to create hydrophobic islands of silanes. Post vapor deposition of the hydrophobic silane through the open regions of the stencil, the remaining uncovered regions on the oxidized polystyrene substrates will be modified with a hydrophilic silane (polyethylene glycol-silane) of varying molecular weight (1200 – 5000 MW range); the specific MW of the PEG-silane will be dictated by the hydrophobic silane in use. The hydrophobic islands of silanes surrounded by the hydrophilic 'sea' of PEG-silane will be primed with cell adhesive extracellular matrix protein (fibronectin, laminin, or collagen) at ~ 25 μg/ml or the cell adhesive tri-peptide RGD at similar concentration. The protein or peptide will bind to the hydophobic regions (hexadecane silane) while resisting adsorption onto the hydrophilic regions (PEG-silane). This in turn will convert the hydrophobic regions into 'cytophilic' regions that are conducive for binding cells.

Cell Seeding and Differentiation on Chips. We will use identical methods for seeding and differentiating cells on the polystyrene and glass chips. The transfected cells will be seeded onto the chips in appropriate medium and incubated for 2 hours, followed by a gentle rinse to remove non-adherent cells settled on the PEG-thiol regions. The cells will be refreshed with medium and incubated overnight. The seeding density will be optimized to enable maximal coverage of the cytophilic islands with cells. Chips bearing SNB-19, NT2, and co-cultures of the two cell types will be homogeneously treated with the differentiating agent(s) by applying the solution homogeneously to every region of the chips. For the special case of chips bearing C2C12 cells and requiring differentiation of cells in selective regions of the chip into either myocytes or adipocytes, we will use a programmable multispotter (Hamilton) custom fitted with a head that matches the volume dispension required for our purpose (50 nl). We will treat each chip either homogeneously with one drug compound (the multiple arrays will be treated as replicates), or microspot several compounds in defined regions on the chip to enable multiple compound testing on a single chip that bears either single or multiple cell types. In parallel, outside of this proposal, we will be forming an alliance with a major microfluidics company to develop a high density microfluidic cassette that will enable addressing each cytophilic island with a reagent of choice.